CD66a CD66b CD66c CD66d CD66e

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Our research group focuses on a group of glycoproteins called CD66 antigens. CD66 consists of five different glycoproteins with similar structures, CD66a, CD66b, CD66c, CD66d and CD66e, which are encoded by the carcinoembryonic antigen (CEA) gene family members, BGP, CGM6, NCA, CGM1 and CEA, respectively. These CD66 antigens are expressed mainly in granulocytes, normal epithelial cells of the digestive tract and tumor cells of various tissues.

Appendix 2

Induction of delayed-type hypersensitivity responses by monoclonal anti-idiotypic antibodies to tumor cells expressing carcinoembryonic antigen and tumor-associated glycoprotein-72

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Abstract. The use of anti-idiotypic antibodies as immunogens represents one potential approach to active specific immunotherapy of cancer. Two panels of syngeneic monoclonal anti-idiotypic antibodies were generated. One panel was directed against mAb CC49 and the other to mAb COL-1. mAb CC49 recognizes the pancarcinoma antigen (Ag), tumor-associated glycoprotein-72 (TAG-72), and mAb COL-1 recognizes carcinoembryonic antigen (CEA). Seven anti-idiotypic (AI) antibodies (Ab2) designated AI49-1-7 were generated that recognize the variable region of mAb CC49. These mAb were shown to inhibit the interaction of mAb CC49 (Ab1) with TAG-72 (Ag). Five anti-idiotypic antibodies designated CAI-1-5 were also generated to the anti-CEA mAb, COL-1 (Ab1). These Ab2 were shown to inhibit the interaction between COL-1 (Ab1) and CEA (Ag). Immunization of mice, rats, and rabbits with Ab2 directed against CC49 or COL-1 could not elicit specific Ab3 humoral immune responses, i.e., antibody selectively reactive with their respective target antigens. However, immunization of mice with the CC49 anti-idiotypic antibody (Ab2), designated AI49-3, could induce a delayed-type hypersensitivity response (DTH) specific for tumor cells that express TAG-72. Similarly, immunization of mice with an anti-idiotypic antibody directed against COL-1, designated CAI-1, could induce specific DTH cell-mediated immune responses to murine tumor cells that express human CEA on their surface. These results thus demonstrate that while some anti-idiotype mAb may not be potent immunogens in eliciting Ab3 humoral responses, they are capable of eliciting specific cellular immune responses against human carcinoma-associated antigens. This type of mAb may ultimately be useful in active immunotherapy protocols for human carcinoma.

Introduction

Among the more studied human carcinoma-associated antigens are carcinoembryonic antigen (CEA) [14] and tumor-associated glycoprotein-72 (TAG-72) [5]. CEA is a 180-kDa glycoprotein expressed on the majority of colon, rectal, stomach, and pancreatic tumors [38], and 50% of breast carcinomas [48] and 70% of lung adenocarcinomas [53]. CEA is also expressed on normal colonic epithelium. TAG-72, initially defined by monoclonal antibody B72.3, is a 10^6-kDa mucin found on the cell surface of colorectal, gastric, pancreatic, ovarian, endometrial, mammary, and non-small-cell lung carcinomas [5]. TAG-72 is not appreciably expressed on a range of normal human tissues with the exception of normal secretory endometrium [49] and transitional mucosa, the mucosa adjacent to the tumor mass [55]. Monoclonal antibodies to TAG-72 [6, 10, 32] and to CEA [2, 15, 45] have both had success in radiolocalization of tumors in patients. Both of these antigens represent potential targets of active specific immunotherapy.

The use of anti-idiotypic antibodies as an immunogen represents one potential approach of active specific immunotherapy. The internal-image anti-idiotypic antibody (Ab2) that recognizes the paratope or the idiotype (Ab1) may mimic the antigenic determinant recognized by the idiotype. Since these complementary idiotypic and anti-idiotypic interactions may function to regulate immune responses [20], an Ab2 can potentially be utilized as a surrogate immunogen to induce specific immune responses [40]. These idiotype/anti-idiotype network systems have also been shown to play a role in the regulation of T cell immunity via immunoglobulin and T cell receptor idiotypes [9, 41, 42].

Traditionally, most investigators characterize the internal image of anti-idiotypic antibodies by the following criteria: (a) Ab2 must be able to bind to the Ab1 idiotype, (b) Ab2 must inhibit Ab1 binding to antigen and (c) Ab2 must be able to induce an anti-anti-idiotype or "Ab3" immune response reactive with the antigen, which could be either a cell-mediated or humoral immune response. Recently, the use of anti-idiotypic antibodies as a vaccine has been reported to produce protective immunity against parasites, bacteria, and viral infections [16, 34, 43]. This strategy is also being pursued in tumor antigen systems for carcinoma [17, 18, 29, 52], melanoma [22, 28] and sarcoma [7] in both rodent [7, 42] and human [17, 22, 28, 29] systems.

We present data here on the generation and characterization of two panels of monoclonal anti-idiotypic antibodies that recognize the variable regions of mAb CC49 and COL-1, that react with TAG-72 and CEA, respectively. CC49 and COL-1 mAb were selected because of their defined pattern of reactivity specific for certain types of human tumors versus the vast majority of normal tissues [38]. COL-1 mAb has also been shown to react specifically with CEA but not with the closely related molecule non-specific cross-reacting antigen that is found on the surface of human granulocytes. COL-1 has a K_a of 1.36×10^9 M^{-1} and has been shown to react with a protein epitope on CEA [26]. mAb CC49 has a K_a of 16.2×10^9 M^{-1} and has been shown to react with a carbohydrate epitope [27]. In ongoing clinical trials, radiolabeled forms of both mAb CC49 and COL-1 have been shown to localize carcinoma selectively in colon cancer patients (S. Larson, and B. Yu, personal communication).

We report here that both sets of anti-idiotypic antibodies could specifically inhibit Ab1 binding to their antigen. However, when the Ab2 were used as an immunogen, an Ab3 humoral immune response of antibodies reactive with the original antigen could not be detected. We do demonstrate, however, that one of the anti-idiotypic antibodies that recognizes the anti-TAG-72 mAb CC49, could induce delayed-type hypersensitivity (DTH) responses specific for tumor cells that express TAG-72. One COL-1 anti-idiotypic antibody, CAI-1, could similarly elicit DTH responses to CEA on the surface of murine tumor cells.

Materials and methods

Animals. Balb/c and C57BL/6 female mice between 6 and 12 weeks old, Wistar rats and New Zealand rabbits were obtained from the Frederick Cancer Research Facility (FCRF), quarantined and maintained in the Health Center Animal Resources Facility at NIH. Female athymic mice (nu/nu) with Balb/c background, also obtained from the FCRF, were used to induce hybridoma ascites in this study.

Cells. The LS-174T colon carcinoma cell line [50], obtained from the American Type Culture Collection (ATCC, Rockville, Md.) was grown as described previously [39]. The MRC-5 human embryonic fibroblast cell line was acquired from the ATCC and maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% heat-inactivated fetal calf serum (FCS). The MC-38 murine colon adenocarcinoma cell line was a gift from Dr. S. Rosenberg [11]. The MC-38 line transduced with the CEA gene (MC-38-CEA-2) was obtained from Dr. P. Robbins

[44]. Both lines were maintained in DMEM containing 10% FCS. The OVCAR-3 human ovarian carcinoma cell line obtained from Dr. David Segal was maintained in ascites of nude mice on a Balb/c background. The tumor cells were harvested from ascites and grown in RPMI-1640 complete medium supplemented with 2 mM glutamine, 1 mM sodium pyruvate, fungizone (0.25 µg/ml), streptomycin (50 µg/ml) and 15% heat-inactivated FCS. All murine hybridoma cell lines were passaged in RPMI-1640 complete medium as described above. Cells were cultured at 37°C in a humidified incubator containing 7.0% CO$_2$. Monolayers were detached from culture flasks with 0.1% trypsin containing 0.5 mM EDTA.

Monoclonal antibodies. Two panels of mouse mAb recognizing the two distinct human tumor-associated antigens, TAG-72 and CEA, were used. One panel of anti-TAG-72 mAb (B72.3, CC11, 15, 29, 30, 40, 46, 49, 83, 92, and 112) recognized multiple epitopes of the TAG-72 molecule [27]. The panel of anti-CEA mAb (COL-1, 4, 6, 7, and 11), was generated as previously described, and recognized different epitopes of CEA [26]. D612 mAb was utilized as an isotype-matched control for these studies. D612 has been reported to react with human gastrointestinal carcinoma and to normal gastrointestinal tissue. It is non-reactive with CEA [39]. A rat monoclonal anti-idiotypic antibody specific for mAb B72.3, designated AI72.3, was utilized as a control for the CC49 Ab2 fine-specificity studies.

Production of anti-idiotypic mAb species (Ab2) to CC49 (Ab1) and COL-1 (Ab1). Balb/c mice were immunized by intraperitoneal (i.p.) and subcutaneous (s.c.) injections of 50 µg/200 µl of either CC49 or COL-1 purified mAb coupled to keyhole limpet hemocyanin (KLH) (Sigma, St. Louis, Mo.) emulsified in complete Freund's adjuvant (Sigma, St. Louis, Mo.) as previously described [42]. Animals were then boosted weekly with the immunogen emulsified in incomplete Freund's adjuvant. The initial boost contained 50 µg/200 µl whereas subsequent boosts contained 20 µg. Prior to fusion, the mice were given a final intravenous (i.v.) boost of 10 µg CC49/KLH or COL-1/KLH conjugates diluted in 100 µl phosphate-buffered saline (PBS). The fusion was performed 3 days later according to the standard methods for hybridoma technology [19]. Briefly, the splenic lymphocytes derived from the mice immunized with either CC49 or COL-1 mAb were harvested and mechanically dispersed over a wire mesh screen (Bellco Cage Products, Boston, Mass.). Subsequently, these cells were fused with the non-immunoglobulin-secreting mouse myeloma cell line P3-NS1 Ag4 ([23], ATCC no. TIB-18) using a 50% solution of polyethylene glycol 1500 (BDH Chemicals Ltd., Poole, England) and cultured in hypoxanthine/aminopterin/thymidine selection medium as previously described [38].

Screening of anti-idiotype hybridoma supernatants. Initial screening of the CC49 Ab2 hybridoma supernatants was done by a solid-phase enzyme-linked immunosorbent assay (ELISA) using a modification of an indirect method for the detection of bound immunoglobulin [42]. A sample containing 50 ng purified CC49 F(ab')$_2$ or a purified preparation of murine polyclonal IgG F(ab')$_2$ fragments diluted in PBS was coated to each well of 96-well polyvinyl chloride flat-bottom microtiter plates (Dynatech, Chantilly, Va.) and incubated overnight at 4°C. For every immunoassay described in this section, antigen-coated microtiter wells were treated with 100 µl 5% bovine serum albumin (BSA, Sigma, St. Louis, Mo.) in PBS for 1 h at 37°C to prevent the non-specific binding of antibody to the plates. A 50-µl sample of either a 1:2 dilution of hybridoma tissue-culture supernatants or varied dilutions of purified Ab2 mAbs was then added to each well. Following a 1-h incubation, 50 µl horseradish-peroxidase-conjugated rabbit anti-(mouse IgG Fc) serum (Jackson Immunoresearch Laboratories, West Grove, Pa.) was added (1:2500) to each well for 1 h, 37°C. After a wash step, any remaining bound immunoglobulin was revealed by a 15-min incubation with 100 µl substrate solution containing 0.013% H$_2$O$_2$ and 2.8 mM o-phenylenediamine dihydrochloride substrate (Sigma, St. Louis, Mo.) diluted in 0.1 M phosphate/citrate buffer, pH 5.0. The reactions were stopped by addition of 25 µl 4 M H$_2$SO$_4$. Plates were read on a Bio-tek microplate ELISA reader EL310 (Winooski, VT) at an absorbance of 490 nm. Those hybridoma supernatants that contained mAb that specifically recognized

CC49 F(ab')₂ fragments but not murine F(ab')₂ fragments were selected for further characterization of purified anti-CC49 mAb.

The COL-1 Ab2 hybridoma supernatants were screened using an inhibition solid-phase radioimmunoassay (SPRIA) where the supernatants were tested for the presence of immunoglobulin that could inhibit ^{125}I-radiolabeled CEA (International Enzyme, San Diego, Calif.) from binding to COL-1 mAb (Ab1) but not inhibit binding to an isotype-identical anti-CEA mAb, COL-4 (IgG2a). In this assay, COL-1 and COL-4 mAb were coated overnight to each well of round-bottom microtiter plates (100 ng/50 μl) at 4°C. The plates were blocked from non-specific protein binding with 100 μl 5% bovine serum albumin (BSA) diluted in PBS for 1 h at 37°C. Samples containing 50 μl hybridoma supernatants at a 1:2 dilution were incubated for 1 h at 37°C. Following the wash step, which removed unbound immunoglobulin, ^{125}I-labeled CEA (50000 cpm/25 μl) was added to each well for 1 h at 37°C. The plates were washed and exposed overnight at –70°C to Kodak XAR film with a lightning-plus screen (Dupont, Wilmington, Del.). Idiotype-specific supernatants were selected on the basis of their ability to inhibit the labeled CEA from binding to COL-1 mAb but not to COL-4 mAb.

Selection of the CC49 Ab2 that inhibit Ag : Ab1 interaction. An inhibition assay was developed to characterize the anti-idiotypic antibodies to CC49 mAb. Purified TAG-72 (0.349 unit/50 μl) was dried down overnight at 37°C to each well of round-bottom microtiter plates (Dynatech, Chantilly, Va.). TAG-72 was purified as previously described from LS-174T colon carcinoma xenografts [46]. One unit of purified TAG-72 is defined as the amount of TAG-72 found in one microgram of a standard tumor extract expressing TAG-72 [21]. In separate microtiter reaction plates that contain no antigen, 10 ng purified CC49 protein (50 μl) was coincubated with 50 μl of either dilutions of Ab2 tissue-culture supernatants or various concentrations of purified immunoglobulin for 1 h at 37°C. Next, 50 μl mixture was transferred to the TAG-72 detection plates and incubated for 1 h at 37°C. Bound CC49 mAb was detected with ^{125}I-radiolabeled goat anti-(mouse IgG H+L chain) specific antisera (75000 cpm/25 μl) (Becton-Dickinson, San Jose, Calif.). The percentage inhibition was calculated by the following formula:

$$100 - \left\{ \frac{100 \times [\text{test sample } ^{125}\text{I (cpm)} - \text{background } ^{125}\text{I (cpm)}]}{\text{total } ^{125}\text{I (cpm)} - \text{background } ^{125}\text{I (cpm)}} \right\}$$

The hybridoma cells that secreted mAb that interfered with CC49 mAb (Ab1) binding to purified TAG-72 were cloned twice and injected into mice for ascites production.

Isotyping of monoclonal anti-idiotypic antibodies. A SPRIA was performed as described previously for the isotype determination of these Ab2 [38].

Purification of anti-idiotypic monoclonal antibodies (Ab2). For the purification of the anti-idiotypic antibodies to CC49, the immunoglobulin was precipitated from the ascites fluid with 40% saturated ammonium sulfate at 4°C for 3 h. The immunoglobulin was then dialyzed overnight against 20 mM TRIS/HCl (pH 7.0) and applied to an ion-exchange column (SAX protein DEAE; Waters, Division of Millipore, Marlborough, Mass.) by high-performance liquid chromatography. Antibody was eluted with a salt gradient ranging from 0 to 0.5 M NaCl diluted in 20 mM TRIS/HCl (pH 7.0). Fractions containing the anti-idiotype mAb were analyzed by sodium dodecyl sulfate/polyacrylamide gel electrophoresis followed by Coomassie blue staining to reveal protein bands. Each fraction was assayed for reactivity by the indirect anti-idiotype binding assay to CC49 F(ab')₂. The fractions containing anti-idiotype mAb were pooled and dialyzed extensively against PBS. The protein concentration was determined by the method of Lowry et al. [31].

The anti-idiotypic antibodies reactive with COL-1 were purified over a column containing *Staphylococcus aureus* protein-A-Sepharose CL4B (SPA-Sepharose) beads (Pharmacia, Uppsala, N.Y.). A 1.5-g sample of dry SPA-Sepharose beads was swollen in 0.1 M NaPO₄ buffer, pH 8.0, for 30 min and 1–2 ml murine hybridoma ascites fluid was added to the beads and rotated at room temperature for 30 min. Following extensive washing with the 0.1 M NaPO₄ buffer, pH 8.0, bound immunoglobulin was eluted with 0.1 M sodium citrate buffer pH 3.0–4.5. Purified immu-

noglobulin was immediately neutralized with 1 M TRIS and dialyzed against PBS. The fractions were characterized and pooled as described above.

Radiolabeling of monoclonal antibodies with iodine-125. The mAb COL-1, CC49, AI49-1-7, CAI-3, and CEA, were labeled with sodium iodide (Na^{125}I) using a modification of the Iodogen technique [12]. Iodogen (Pierce Chemical, Rockford, Ill.) was diluted in chloroform to 10 mg/ml and 20-ml aliquots were evaporated under a stream of nitrogen and stored at –20°C until use. A 50-μg aliquot of antibody or 200 μg antigen diluted in PBS and 0.5 mCi Na^{125}I were added to the iodogen tubes. After a 2-min incubation at room temperature, the protein was removed from the insoluble iodogen and the unincorporated ^{125}I was separated from the antibody by gel filtration through Sephadex G-25 (Pharmacia Fine Chemicals, Piscataway, N.J.).

Antibody coupling with KLH. Monoclonal antibodies were coupled to the carrier protein keyhole limpet hemocyanin (KLH, Sigma Chemical, St. Louis, Mo.) by chemical cross-linking in the presence of glutaraldehyde (Sigma Chemical, St. Louis, Mo.) as described [33].

Ab2 induction of the Ab3 humoral response. Balb/c mice, Wistar rats and New Zealand white rabbits were immunized with anti-idiotypic antibodies AI49-1-6 to examine the specificity of the humoral Ab3 immune response within and across species boundaries. Wistar rats were immunized with mAb AI49-3, 4 and 5. Three animals per group of mice and rats were immunized subcutaneously with either 25 μg or 50 μg purified anti-idiotypic antibodies coupled to KLH and emulsified in complete Freund's adjuvant. Subcutaneous boosts of the same amount of immunogen emulsified in incomplete Freund's adjuvant were administered every 2 weeks. Rabbits were immunized subcutaneously at multiple sites with 50 μg anti-idiotypic antibodies coupled to KLH, also emulsified in adjuvant as described above. All animals were bled every 2 weeks either 7 days following each boost for mice and rats or just prior to each boost for the rabbits. Two strains of mice (Balb/c and C57BL/6) and New Zealand white rabbits were also immunized as described above with three of the anti-idiotypic mAb to CEA designated CAI-1, CAI-3 and CAI-5.

Quantification of TAG-72-reactive antibody. Serum samples from mice and rats were collected from the tail vein into Natelson heparanized collecting tubes (Government Marketing Services, Washington, D.C.). All serum samples were tested in a modified SPRIA for antibody reactivity to purified TAG-72. TAG-72 (0.054 U/50 μl) and BSA (100 ng/50 μl) diluted in PBS were each dried down to microtiter wells overnight at 37°C. Serial dilutions of serum starting at 1:100 were incubated with the antigen on the plate for 1 h at 37°C. For the detection of bound mouse immunoglobulin, ^{125}I-labeled goat anti-(mouse IgG H+L chain) specific antisera (75000 cpm/25 μl) was incubated for 1 h at 37°C. Bound rat immunoglobulin was determined utilizing a rabbit anti-(rat IgG H+L chain) specific linker (1:2500) (Jackson Immunoresearch Laboratories, West Grove, Pa.) followed by ^{125}I-labeled *S. aureus* protein A (SPA) (50000 cpm/25 μl). Rabbit antiserum binding to TAG-72 or BSA was detected by ^{125}I-labeled SPA (50000 cpm/25 μl).

Quantification of antibodies reactive with CEA. Samples of mouse and rabbit sera were quantified for anti-CEA antibodies by ELISA. Microtiter plates were dried down overnight at 37°C with 100 ng/well purified CEA. The wells were incubated with dilutions of mouse or rabbit antiserum, preimmunization serum, or the anti-CEA mAb, COL-1. Bound antibody was detected with horseradish-peroxidase-conjugated goat anti-(mouse IgG) antiserum or similarly conjugated donkey anti-(rabbit IgG) antiserum (Becton Dickinson, San Jose, Calif.). The complex was detected using the o-phenylenediamine chromogen as described above.

Ab2 induction of antigen-specific delayed-type hypersensitivity responses. The induction of cell-mediated immunity to TAG-72 by immunization with anti-idiotypic mAb AI49-1-7 was explored using a delayed-type hypersensitivity assay [23]. Balb/c mice were immunized twice at 2-week intervals with 1.5×10^7 irradiated (40 Gy) hybridoma
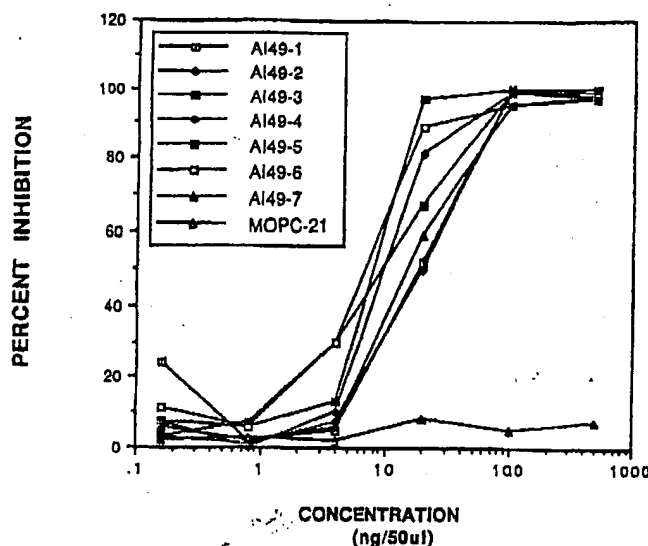
Fig. 1. Inhibition of CC49 mAb (Ab1) binding to TAG-72 by Ab2 species. Purified TAG-72 (0.35 U/50 μl/well) was dried down overnight, 37°C, to microtiter plates. In a separate reaction plate with no antigen bound, purified CC49 mAb (20 ng/50 μl) was coincubated with fivefold dilutions of anti-idiotypic mAb AI49-1–7, or control IgG, MOPC-21, starting at 1000 ng/50 μl for 1 h, 37°C. Samples containing 50 μl mixture were then incubated on the TAG-72 detection plate for 1 h. Bound CC49 mAb was detected by a subsequent 1-h incubation with ^{125}I-radiolabeled goat anti-(mouse IgG) (heavy- and light-chain-specific) antisera (75000 cpm/25 μl). The percentage inhibition was calculated as described in Materials and methods

cells secreting the anti-idiotypic antibody, AI49-3, or human tumor cells that express TAG-72, emulsified in incomplete Freund's adjuvant (IFA). X-irradiated hybridoma cells secreting a control isotype-matched mAb (COL-12) emulsified in IFA, PBS emulsified in IFA, and PBS alone were utilized as control immunogens. In a separate experiment, Balb/c mice were immunized with 100 μg purified anti-idiotypic antibodies AI49-3–5 coupled to KLH in the manner described above. Mice were challenged with an injection in one footpad of 5×10^5 X-irradiated human ovarian carcinoma cells (OVCAR-3), which express TAG-72, 7 days following the final boost. As a control for a non-specific DTH response, each mouse received an injection of 5×10^5 X-irradiated MRC-5 human fibroblast cells in the opposite footpad and expressed in "mil" (0.0254 mm). After 48 h, footpad thickness was measured with a micrometer. DTH was calculated as the difference of footpad swelling between hind footpads. This experiment was repeated four times with three to five mice per group and readings were made in a blind manner.

C57BL/6 mice were also utilized to analyze the specific DTH responses induced by the purified COL-1 anti-idiotypic antibodies. In three separate experiments, four to six mice per group were immunized intraperitoneally with X-irradiated hybridoma cells (40 Gy) secreting COL-1 Ab2, CAI-1, or X-irradiated hybridoma cells secreting a control immunoglobulin D612. Seven days following the last immunization, 5×10^5 X-irradiated (200 Gy) human-CEA-transduced murine tumor cells, MC-38-CEA-2, in 20 μl PBS were injected into one hind footpad and 20 μl PBS containing 5×10^5 X-irradiated cells from the non-transduced cell line, MC-38, were injected into the other hind footpad. The thickness of the footpads was measured 48 h later as described. The P values were determined utilizing Student's t-test of significance [47].

Results

Generation of anti-idiotypic antibodies to mAb CC49

Spleens from mice immunized with CC49 mAb coupled to KLH were subsequently harvested for hybridoma production. The supernatants from a total of 2750 wells were screened in a solid-phase ELISA for the presence of antibodies that bound to CC49 F(ab')₂ fragments versus control murine polyclonal F(ab')₂. Out of 2750 wells screened, 26 were reactive with CC49 F(ab')₂ but not with the control murine F(ab')₂ fragments. The remaining wells were negative to both CC49 and control F(ab')₂. No mAb were generated that recognize both CC49 and the control IgG F(ab')₂.

In order to determine whether these hybridoma supernatants contain immunoglobulin reactive with sites associated with the paratope of mAb CC49, the CC49-reactive supernatants were screened in a competition radioimmunoassay. Out of 26 anti-idiotypic antibody supernatants, 10 inhibited mAb CC49 from binding to TAG-72. These Ab2 represent the portion of the total population that could be classified as potential Ab2 that may bear the image of an epitope on TAG-72. Seven of the wells containing anti-idiotypic antibodies to mAb CC49 that most efficiently inhibited CC49 binding to TAG-72 were selected for ascites production and further characterization. These mAb were designated AI49 (anti-idiotypes to CC49) 1–7.

Binding reactivity of purified anti-idiotypic antibody, AI49-1–7.

Studies were undertaken to determine if the purified Ab2 species, AI49-1–7, could inhibit mAb CC49 from binding to the TAG-72 antigen. As seen in the radioimmunoassay results shown in Fig. 1, all of the purified Ab2 specifically inhibited mAb CC49 (Ab1) from binding to purified TAG-72 while the irrelevant control antibody, MOPC-21, failed to inhibit binding. These results suggested that the purified anti-idiotypic antibodies recognize site(s) proximal to the CC49 antigen combining site.

Specificity of monoclonal anti-idiotypic antibodies (Ab2) for a site unique to mAb CC49 (Ab1).

In a previous report [27], we demonstrated that many of the anti-TAG-72 "CC" mAb, including CC49, were shown to cross-compete with each other in a reciprocal competition radioimmunoassay. A radioimmunoassay was designed to determine if the Ab2 species AI49-1–7 recognize determinants common to some or all of a panel of ten anti-TAG-72 mAb, or only recognize a determinant unique to the mAb CC49. AI49-1–7 were radiolabeled and tested in direct-binding radioimmunoassay for immunoreactivity to a panel of CC mAb. As shown in Table 1, the radiolabeled AI49-1–7 mAb species bound uniquely to the mAb CC49 idiotype but not to any of the other idiotypes on the anti-TAG-72 mAb species or the irrelevant control mAb, COL-3. As a positive control, ^{125}I-labeled goat anti-(mouse IgG) antiserum was shown to bind to every well containing IgG. Therefore, mAb AI49-1–7 recognize epitopes restricted to CC49 among the panel of CC mAb.

Table 1. Binding reactivity of anti-idiotypic antibodies (Ab2) AI49-1–7 to a panel of anti-TAG-72 mAb[a]

mAb (Ab1)	Isotype	^{125}I-labeled AI49 mAb (Ab2) (cpm)							
		AI49-1	AI49-2	AI49-3	AI49-4	AI49-5	AI49-6	AI49-7	GAM
B72.3	IgG1	65	79	63	79	19	45	109	4276
CC11	IgG1	64	36	104	45	24	0	54	5430
CC15	IgG2b	73	42	54	35	133	4	194	4859
CC29	IgG1	89	0	69	54	57	8	95	4174
CC40	IgG1	79	35	63	99	63	4	105	3238
CC46	IgG1	79	54	68	68	40	149	155	4851
CC49	IgG1	3106	12056	9110	6938	10072	3597	3498	2475
CC50	IgG1	160	47	130	78	4	15	82	5348
CC83	IgG1	175	89	64	184	79	2	34	4304
CC112	IgM	135	57	74	75	29	1	147	283
COL-3	IgG1	104	57	49	55	47	4	89	3569
PBS	NA[b]	176	25	19	95	31	5	53	24

[a] Microtiter plates were coated overnight at 4°C with 50 ng/50 μl different mAb. Following an incubation with phosphate-buffered saline (PBS) containing 5% bovine serum albumin to block non-specific protein binding, ^{125}I-radiolabeled AI49 anti-idiotypic antibodies or ^{125}I-radiolabeled

goat anti-(mouse IgG) (GAM) (50000 cpm/well) were incubated on the coated microtiter plates for 1 h. Plates were washed and bound immunoglobulin was determined as cpm bound

[b] NA, not applicable

Table 2. Fine binding specificity of anti-idiotypic antibodies (Ab2), AI49-1–7, for the CC49 idiotype (Ab1)[a]

Inhibitor Ab2 mAb	Inhibition 50% (ng)[b]					
	AI49-3	Index-3[c]	AI49-5	Index-5	AI49-6	Index-6
AI49-1	2.4	3.8	2.5	0.9	5.0	7.8
AI49-2	7.8	12.2	2.7	1.0	6.5	10.2
AI49-3	0.6	1.0	<0.6	<0.2	1.5	2.3
AI49-4	105.0	164.1	5.0	1.9	12.5	19.5
AI49-5	16.0	25.0	2.7	1.0	7.0	11.0
AI49-6	9.0	14.1	<0.6	<0.2	0.6	1.0
AI49-7	5.5	8.6	<0.6	<0.2	3.8	5.9
AI72.3	2000.0	3125.0	2000.0	>741.0	2000.0	3125.0

[a] Microtiter wells coated with CC49 F(ab')$_2$ fragment (50 ng/50 μl) were incubated with fivefold dilutions of different Ab2 or the irrelevant control anti-idiotypic antibody to B72.3, AI72.3, for 1 h at 37°C. Subsequently, ^{125}I-radiolabeled AI49 Ab2 mAb, AI49-3, AI49-5 and AI49-6, were added to the antigen plate (75000 cpm/25 μl) and the mixture was incubated overnight, 4°C. The percentage inhibition was calculated as described

[b] Quantity (ng) required to inhibit ^{125}I-radiolabeled Ab2 binding by 50% (I_{50})

[c] Index the experimental competitor I_{50} values divided by the I_{50} value obtained by an Ab2 competing with itself

Fine specificity of the AI49 mAb (Ab2) binding to the CC49 idiotype (Ab1). Reciprocal antibody competition radioimmunoassays were designed to "map" the binding location of the different Ab2 species, AI49-1–7, to the idiotype of mAb CC49 and to delineate whether fine binding differences exist between them. Table 2 summarizes the data from these mapping studies. The panel of Ab2 was analyzed for their ability to inhibit radiolabeled Ab2 from binding to mAb CC49 (Ab1); AI49-3, 5, and 6 were radiolabeled for this study. For these studies, complete inhibition curves were generated for each competitor Ab2 and the quantity (ng) required to inhibit the radiolabeled Ab2 by 50% (I_{50}) was determined. From these values, an index

was derived by dividing the experimental competitor I_{50} values by the I_{50} value obtained by an Ab2 competing with itself. The relatively low indices indicate that all of the Ab2 mAb could efficiently inhibit the labeled anti-idiotypic antibodies from binding to mAb CC49 (Ab1) (Table 2). A control anti-idiotypic antibody AI72.3, which does not react with CC49, failed to compete for binding.

Fine differences in binding were noted between the Ab2 species (Table 2). AI49-4 required 164-fold more antibody for 50% inhibition than the homologous competitor AI49-3, indicating that it may recognize a related but different epitope on mAb CC49 or may have a lower affinity than that of AI49-3. AI49-5 appeared to have a lower affinity to mAb CC49 compared to the other types of Ab2. In some cases (AI49-3, 6, 7), less competition antibody was required to cause 50% inhibition than when AI49-5 was used as a competitor against itself. mAb AI49-2, 4, 5 appear to recognize related but different epitopes as AI49-6.

Generation of monoclonal anti-idiotypic antibodies to carcinoembryonic antigen

Spleens from mice immunized with COL-1 mAb coupled to KLH were harvested for hybridoma production. Hybridoma supernatants from 5000 wells were screened in a solid-phase competition radioimmunoassay for the presence of antibody that could inhibit ^{125}I-radiolabeled CEA from binding to either mAb COL-1 or isotype-identical mAb COL-4. Five supernatants out of 5000 wells screened (0.1%) contained immunoglobulin that specifically inhibited radiolabeled CEA binding to COL-1 but not to mAb COL-4. These five anti-idiotypic antibodies were designated the COL-1 anti-idiotypic antibodies, CAI-1–5. No supernatants were observed that would inhibit CEA from binding to mAb COL-4.
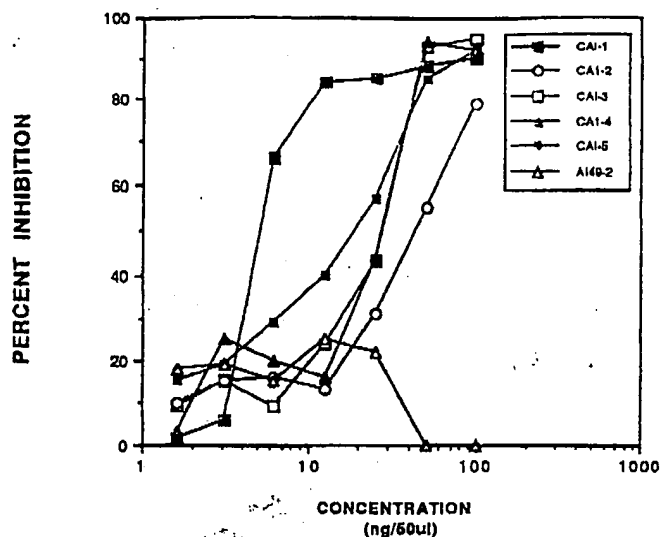
Fig. 2. Inhibition of COL-1 (Ab1) binding to carcinoembryonic antigen (CEA) (Ag) by Ab2. Purified CEA (25 ng/well) was dried down overnight to each well on microtiter plates. In a separate blank reaction plate, twofold dilutions of the purified anti-idiotypic antibodies, CAI-1 (■), CAI-2 (O), CAI-3 (□), CAI-4 (▲), CAI-5 (⊞) as well as control mAb, AI49-2 (△) (starting at 100 ng/25 µl) were coincubated with ^{125}I-radiolabeled COL-1 mAb (25 000 cpm/25 µl) for 1 h, 37°C. Samples containing 50 µl mixtures were then incubated for 1 h on the CEA detection plates. The radioactivity (cpm) was detected and percentage inhibition was determined as described in Materials and methods

Fig. 3. Fine specificity of the anti-idiotypic antibodies (Ab2), CAI-1-5 binding to the COL-1 (Ab1) idiotype. Microtiter wells were coated overnight, 4°C, with 50 ng/50 µl purified COL-1 mAb (Ab1). Following a step to block non-specific protein binding, the plates were incubated for 1 h with twofold dilutions of purified anti-idiotypic antibodies, CAI-1 (■), CAI-2 (O), CAI-3 (□), CAI-4 (▲), CAI-5 (⊞) as well as a control immunoglobulin, AI49-2 (△) at a starting concentration of 200 ng/50 µl. Subsequently, ^{125}I-radiolabeled anti-idiotypic mAb, designated CAI-3 (50 000 cpm/25 µl), was added to the plates and incubated overnight at 4°C. The percentage inhibition was calculated as described in Materials and methods

Table 3. Binding specificity of the COL-1 anti-idiotypic antibodies to a panel of anti-(carcinoembryonic antigen) (anti-CEA) monoclonal antibodies[a]

Ab2 Inhibitor TCS	Anti-CEA mAbs (Ab1) (% inhibition)				
	Isotype	COL-1	COL-4	COL-6	COL-7	COL-11
CAI-1	IgG2a	100	10	8	0	17
CAI-2	IgG1	96	1	7	16	23
CAI-3	IgG2a	98	0	8	16	0
CAI-4	IgG2b	99	3	0	0	0
CAI-5	IgG2b	92	5	0	0	0
NS-1	NA[b]	0	0	0	0	0

[a] Microtiter plates were coated with a panel of five anti-CEA monoclonal antibodies (100 ng/50 µl) and each was incubated with Ab2 hybridoma tissue-culture supernatants (TCS) or control TCS from NS-1 myeloma cells for 1 h, 37°C. The plates were washed and incubated for 1 h with ^{125}I-radiolabeled CEA (50000 cpm/25 µl). The percentage inhibition of the radiolabeled CEA binding to the anti-CEA monoclonal antibodies was calculated as described in Materials and methods

[b] Not applicable

Binding reactivity of purified anti-idiotypic antibodies, CAI-1–5.

Studies were undertaken to analyze and compare the binding reactivities of the purified Ab2 species, CAI-1–5. In the competition radioimmunoassay shown in Fig. 2, CAI-1–5, specifically inhibited ^{125}I-radiolabeled mAb COL-1 (Ab1) from binding to purified CEA (Ag). A control mAb directed at the CC49 idiotype failed to inhibit the labeled mAb COL-1 binding. The five different Ab2 demonstrated inhibition curves that had three distinct slopes suggesting that these Ab2 may recognize different
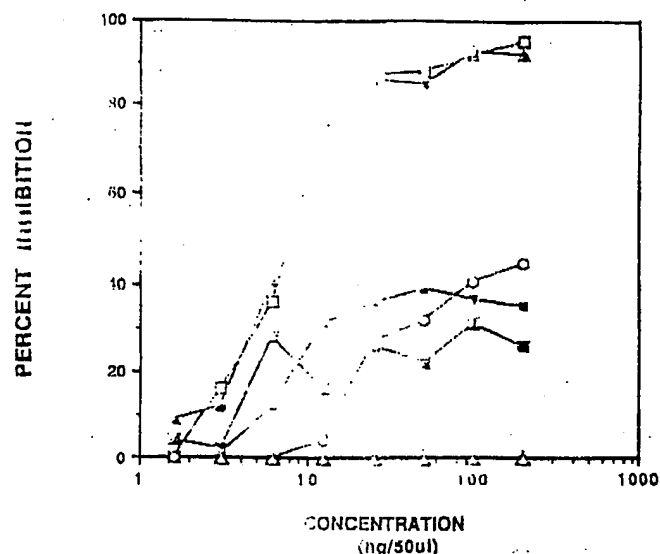
sites on the COL-1 idiotype (Ab1). Ab2 mAb, CAI-1, inhibited COL-1 binding with the highest relative affinity; the mAb required only 4.5 ng to achieve 50% inhibition. Ab2 mAb CAI-3 and CAI-4 demonstrated superimposable curves suggesting that they may recognize very related or identical epitopes. CAI-2 and CAI-5 have similar slopes suggesting that they react with similar epitopes; CAI-5 (Ab2) appeared to have a higher affinity than the rest of the Ab2 CAI-2–4 to mAb COL-1 (Ab1).

Anti-idiotypic monoclonal antibodies, CAI-1–5, recognize a site unique to COL-1. Previous studies using reciprocal competition RIA have shown various degrees of cross-reactivity among the anti-CEA COL mAb series. Specifically, COL-1, 4, 6, 7, and 11 all cross-compete with each other for CEA binding and cannot be distinguished from each other on the basis of these assays [26]. A competition radioimmunoassay was designed to determine if the COL-1 Ab2 mAb recognize determinants found on a panel of anti-CEA mAb. As shown in Table 3, all of the Ab2 inhibited the binding of ^{125}I-radiolabeled CEA to mAb COL-1 species but not the binding of ^{125}I-radiolabeled CEA to any of the other anti-CEA mAb. These data suggested that these Ab2 specifically recognize epitopes unique to the COL-1 idiotype.

Fine binding specificity of anti-idiotypic antibodies CAI-1–5 for the COL-1 idiotype. An antibody competition radioimmunoassay was designed to map the binding of the different Ab2 mAb to the idiotype of the mAb COL-1
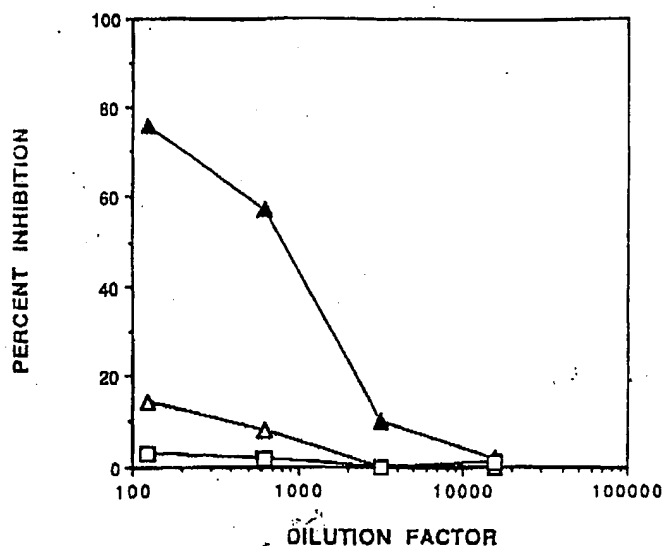
Fig. 4. Reactivity of rabbit serum (Ab3) binding to the CC49 anti-idio-typic antibody (Ab2) immunogen, AI49-5: One rabbit was immunized twice at 2-week intervals with the CC49 Ab2, AI49-5. Fivefold dilutions of preimmunization (△), rabbit anti-AI49-5 serum (▲) and a control hyperimmune serum against another Ab2, AI49-3 (□) (starting at 1:125) were tested for reactivity to purified AI49-5 Ab2. Rabbit anti-(mouse Ig) Ab3 serum was diluted in phosphate-buffered saline (PBS) with 1% mouse serum to absorb out anti-Fc rabbit responses. Microtiter plates coated with purified AI49-5 mAb (100 ng/well) were incubated with different dilutions of rabbit antibodies for 1 h, 37°C. ^{125}I-radio-labeled CC49 mAb (50000 cpm/25 µl) was sequentially added to the plates and incubated for 1 h at 37°C. The percentage inhibition of CC49 mAb binding was calculated as described in Materials and methods

(Ab1) to delineate whether there are fine binding differences between the Ab2 species. Figure 3 illustrates the results of a competition assay in which the panel of Ab2 were analyzed for their ability to inhibit ^{125}I-labeled CAI-3 Ab2 mAb from binding to the COL-1 idiotype (Ab1). As shown, mAb CAI-4 (Ab2) completely inhibited labeled CAI-3 Ab2 binding to COL-1 Ab1, producing a curve superimposable onto the CAI-3 Ab2 inhibition curve of its own binding to COL-1 (Ab1). These data suggested that CAI-3 (Ab2) recognizes the same site as CAI-4 (Ab2) on COL-1 (Ab1). The remaining Ab2 species could only partially inhibit mAb CAI-3 binding, indicating that the Ab2 differ in affinity or that they recognize distinct epitopes on the COL-1 idiotype. The irrelevant control antibody failed to inhibit ^{125}I-CAI-3 binding to mAb COL-1. These results suggest that the CAI Ab2 antibodies can be distinguished by their binding to COL-1.

Analysis of Ab3 humoral immune responses induced by Ab2

Humoral Ab3 immune responses elicited by AI49-1 -6. The anti-idiotypic antibodies to CC49 (i.e., AI49-1-6) were tested for their ability to induce an antigen-specific humoral immune response (Ab3) within and across species barriers. This is the classical criterion to define if anti-idio-typic (Ab2) mAb express the internal image of Ab1, thus

mimicking a B cell epitope on the antigen. These studies were performed in mice, rats and rabbits to analyze Ab3 humoral immune responses. Mice, in groups of three, were immunized with 25 - 30 µg purified Ab2, AI49-1-6, and a control immunoglobulin COL-12. Rats, also in groups of three, were immunized with 15 µg AI49-3-5. One rabbit each was administered 50 µg AI49-1-6. Sera were tested in solid-phase radioimmunoassay for reactivity against TAG-72 and an irrelevant control antigen 14 days following each boost. Sera from all rats and three out of six rabbits were also tested for specific reactivity to the Ab2 immunogen. All sera tested displayed strong titers of antibody reactive with the idiotype of the Ab2 immunogen (Fig. 4). On the other hand, none of the antisera derived from the Ab2-immunized mice, rats, and rabbits gave rise to antibody specific for TAG-72 during the 4-month period of biweekly immunizations.

Ab3 antisera derived from immunized rats and rabbits were analyzed for the presence of anti-Ab2-specific immune responses to ensure the animals were responding to immunization. Figure 4 illustrates representative results from one rabbit immunized with mAb AI49-5. The serum was tested for its ability to inhibit the binding of ^{125}I-radio-labeled CC49 to purified AI49-5 coated on the microtiter plate. AI49-5 Ab3 serum derived 14 days following the second immunization (day 28) demonstrated specific inhibition. On the other hand, the AI49-5 rabbit preimmunization serum and an Ab3 rabbit serum against a different Ab2 (AI49-3) did not compete. These data indicated that the rats (immunized with AI49-3, 4, and 5) and rabbits (immunized with Ab2 AI49-1 - 6) tested in this manner elicited specific Ab3 immune responses to the Ab2 idiotype that was utilized as immunogen. Titers to the Ab2 idiotype were also shown to rise with subsequent immunizations (data not shown).

Humoral Ab3 responses elicited by anti-idiotypic antibodies to COL-1 (CAI-1 - 5). Studies were undertaken to determine whether Ab2 mAb CAI-1-5 express the internal image of the COL-1 idiotype (Ab1) thus mimicking an epitope on CEA (Ag). These studies were done in Balb/c and C57BL/6 (three per group) mice and New Zealand rabbits (one or two per group) to analyze the induction of antigen-specific Ab3 humoral immune responses. All animals were immunized as described in Materials and methods with 50 µg purified Ab2 antibodies coupled to KLH. Preimmunization and serum samples taken 14 days following each boost were tested in ELISA against purified CEA and an irrelevant control antigen, thyroglobulin. None of the Ab2 immune sera demonstrated antibody specifically reactive to purified CEA during the 4-month immunization period.

All of the Ab3 rabbit sera were tested for the presence of antibody reactive with the Ab2 utilized as immunogen. Figure 5 illustrates that the rabbit Ab3 sera obtained 14 days following the second immunization (day 28) contained antibodies specifically reactive with the Ab2 utilized as immunogen but not with the control COL-1 anti-idiotypic antibodies. Specific anti-Ab2 immune responses were noted in all three rabbits tested. The anti-CAI-1 rabbit Ab3 serum was shown to react with purified CAI-1 Ab2 mAb
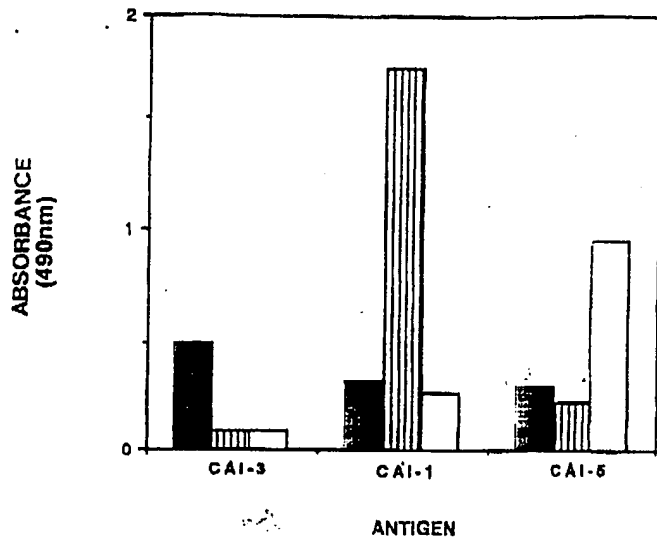
Fig. 5. Reactivity of rabbit Ab3 serum binding to the COL-1 anti-idio-. typic antibodies (Ab2). New Zealand White rabbits were immunized at 2-week intervals with purified COL-1 anti-idiotypic antibodies CAI-1, 3, and 5 coupled to keyhole limpet hemocyanin as described. Rabbit Ab3 sera obtained 14 days after the second immunization were tested for reactivity to their Ab2. Anti-idiotypic antibodies to COL-1 mAb, designated CAI-1, 3 and 5, were coated to microtiter plates (50 ng/well) overnight at 4°C. The rabbit sera against the Ab2 species, CAI-1 (*striped boxes*), CAI-3 (*black boxes*) and CAI-5 (*white boxes*), were diluted in PBS with 1% mouse serum in order to adsorb out anti-(mouse IgG) responses. A 1:3125 dilution of each serum was incubated on the antigen plates for 1 h, 37°C. Following a wash step, the plates were treated for 1 h with horseradish-peroxidase-conjugated *Staphylococcus aureus* protein A. The o-phenylenediamine chromogen was added for 10 min for the detection of the bound IgG complexes
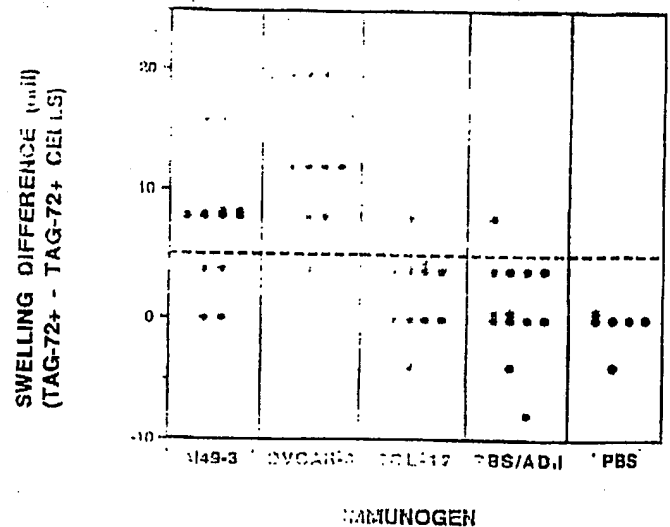

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Fig. 6. Anti-idiotypic antibody (Ab2) induction of delayed-type hypersensitivity (DTH) responses to human tumor cells expressing TAG-72 (Ag). Balb/c mice were immunized with 1.5×10^7 X-irradiated hybridoma cells secreting the anti-idiotypic antibody (AI49-3). Hybridoma cells secreting a control IgG (COL-12). PBS in adjuvant or PBS alone were used as control immunogen. Human ovarian carcinoma cells, OVCAR-3, expressing TAG-72 were immunized as a control for a positive DTH response (TAG-72). Seven days following the last boost, each mouse received an injection of 5×10^5 X-irradiated OVCAR-3 into one footpad. As a control for non-specific swelling, the mice received an injection of 5×10^5 X-irradiated .... negative human embryonic fibroblast cells. MRC-5. In the .... footpad. DTH responses were measured 48 h later and expressed in .... (0.0254 mm) as the difference between footpad swelling. The *dotted line* represents an arbitrary baseline for a positive response. Each *dot* represents one mouse. PBS/ADJ, PBS in adjuvant

but not to purified CAI-5 Ab2 mAb. Likewise, anti-CAI-3 rabbit serum only bound to CAI-3 and anti-CAI-5 only bound to CAI-5.

Induction of cell-mediated immunity by Ab2

Ab2 induction of DTH in tumor cells that express TAG-72. Studies were conducted to determine whether immunization of mice with the AI49 anti-idiotypic antibodies can induce cell-mediated immune responses to TAG-72. Two preliminary DTH experiments were performed in which mice were immunized with X-irradiated hybridoma cells secreting AI49-3, 4, or 5 (see Materials and methods). These were three of the Ab2 species that appeared to differ from each other on the basis of the fine binding specificity competition assays. Ab2 AI49-3 was the only antibody that demonstrated differential swelling in response to challenge with the TAG-72-expressing OVCAR-3 ascites cells in 6 out of 8 animals (mean of 203 μm, 8 "mil") (Fig. 6). In addition, purified anti-idiotypic antibodies, AI49-3, 4 and 5 coupled to KLH were tested at one dose level using the same immunization regimen and did not induce DTH responses (data not shown).

A summary of the DTH results from four experiments is shown in Fig. 6. Utilizing 127 μm (5 "mil") as an arbitrary baseline level of a positive DTH response, 9 out of 13 mice

immunized with the hybridomas secreting Ab2 AI49-3, demonstrated positive swelling responses (Fig. 6). On the other hand, only 2 out of 32 mice immunized with one of the controls (a hybridoma secreting an isotype-matched control IgG emulsified in IFA, PBS emulsified in IFA or PBS alone) demonstrated responses above the baseline for differential swelling. As a control for a positive DTH response, the irradiated OVCAR-3 cells were also used as immunogen as described above. Out of 11 animals, 10 demonstrated a differential DTH response to a footpad challenge with OVCAR-3 and MRC-5 cells respectively. Thus, AI49-3-Ab2-secreting hybridoma cells were able to elicit significant DTH responses to TAG-72-expressing human tumor cells compared to TAG-72-negative human embryonic fibroblast cell line (Fig. 6).

A two-tailed Student's t-test was utilized to analyze the statistical significance of the difference between the means of the observed responses between groups immunized with either the hybridoma cells or the anti-idiotypic antibody or with the control immunogens described above. These calculations were conducted in such a manner to account for variation between individual experiments. Table 4 reports the P values calculated from the combinations of pairwise comparisons of observed responses between the groups of immunogen. AI49-3 significantly induced antigen-specific responses to the TAG-72-expressing ascites cell line. OVCAR-3, when compared to all of the control

Table 4. Statistical analysis of delayed-type hypersensitivity (DTH) responses induced by the CC49 anti-idiotypic antibody, AI49-3[a]

Immunogen: pairwise comparisons				
Group 1 (cells)	Group 2 (cells)	t	(df)[b]	P
AI49-3	Controls[c]	4.150	(39)	<0.001
AI49-3	COL-12	2.880	(20)	0.010
OVCAR-3	Controls	5.949	(37)	<0.001
OVCAR-3	COL-12	3.872	(18)	0.001
PBS/ADJ[d]	COL-12	0.0200	(20)	1.000

[a] Mice were immunized twice with the immunogens AI49-3 hybridoma cells, TAG-72+ OVCAR-3 cells, control hybridoma cells secreting COL-12, PBS emulsified in adjuvant and PBS alone. Seven days following the last immunization, mice were challenged by administering 5×10^5 OVCAR-3 cells in one footpad and 5×10^5 MRC-5 human fibroblast cells in the other footpad. After 48 h, the footpads were measured (see Materials and methods) and DTH was determined as the difference in footpad swelling. A two-tailed Student's t-test of significance was utilized to calculate the P values of the differences of the mean DTH responses between groups immunized with the hybridoma cells secreting the anti-idiotypic antibody (AI49-3) versus those that were immunized with either all of the control immunogen (Controls) or versus only those that received the hybridoma cells secreting an irrelevant immunoglobulin (COL-12). Comparisons were also made between the mean DTH responses observed between groups immunized with OVCAR-3 cells versus the mean response observed between groups immunized with the control immunogen or the hybridoma cells secreting the irrelevant immunoglobulin, mAb COL-12. From the differences between the means, t was calculated and P values were determined [41]
[b] Degrees of freedom
[c] Controls included irradiated hybridoma cells secreting isotype-matched immunoglobulin COL-12, PBS emulsified in Freund's adjuvant, or PBS alone
[d] PBS emulsified in Freund's adjuvant

immunogens (P <0.001) or the control hybridoma COL-12 immunogen alone (P = 0.01). As a positive immunogen, OVCAR-3 induced significant DTH responses compared to all of the control immunogens combined (P <0.001) and to COL-12 hybridoma alone (P <0.001). In contrast, no differences were observed between groups of mice that received PBS emulsified in IFA compared to responses observed in the groups of mice immunized with the COL-12 hybridoma cells (P = 1.0).

Ab2 induction of delayed-type hypersensitivity responses to cells expressing CEA. Studies were done to examine the ability of Ab2 mAb to elicit specific cell-mediated immune responses to CEA on the surface of a tumor cell. Recently we have reported on the generation of a murine C57BL/6 colon adenocarcinoma cell line that has been transduced with the gene for human CEA (MC-38-CEA-2) [44]. The availability of these cells as well as the non-transduced MC-38 cells (CEA-negative) offered an excellent model to test specificity. Figure 7 illustrates the results of a series of three DTH experiments utilizing CAI-1 Ab2 mAb as immunogen. CAI-1 was selected for these experiments because initial experiments demonstrated that CAI-1, out of all of the anti-idiotypic antibodies to COL-1, could elicit DTH responses to the CEA-transduced cells. Data from this experiment aare included in Fig. 7. With an arbitrary
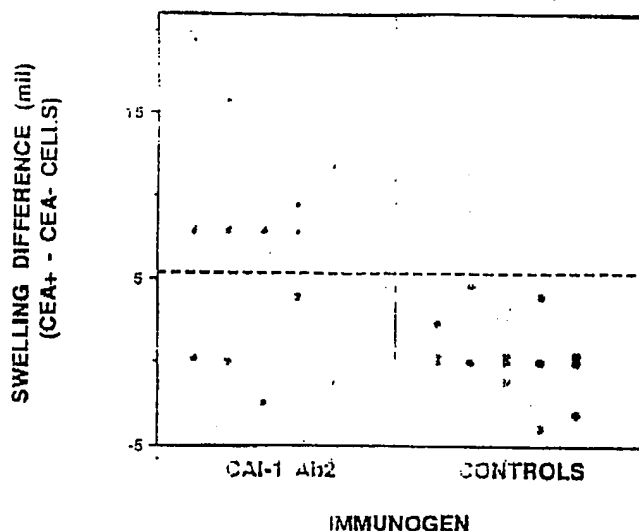

Fig. 7. Anti-idiotypic antibody (Ab2) induction of DTH responses to CEA-transduced murine tumor cells. C57BL/6 mice were immunized as described in the text with X-irradiated hybridoma cells (40 Gy) secreting the anti-idiotypic antibody (Ab2) (CAI-1), or hybridoma cells secreting a control isotype-matched mAb, D612 that is non-reactive with CEA [38]. Seven days following the boost, each mouse was challenged with an injection of 5×10^5 X-irradiated murine tumor cells expressing CEA in one hind footpad. To control for non-specific swelling, 5×10^5 X-irradiated murine non-transduced tumor cells were injected into the opposite footpad. After 48 h, DTH responses were measured in mil (0.0254 mm) as the difference between footpad swelling. The *doned line* is an arbitrary value for a positive response. Each dot represents one mouse

baseline for a positive DTH reaction of 140 μm (5.5 "mil"), 8 out of 13 mice immunized with CAI-1 Ab2 mAb showed significant (P <0.001) responses to the CEA-transduced murine tumor cells (average = 190 μm) compared to the mice receiving the control immunogen (average = 6 μm). These data demonstrate that mAb CAI-1, could induce statistically significant cell-mediated immunity directed against tumor cells expressing human CEA (Fig. 7).

Discussion

This paper explores one approach of active specific immunotherapy, the use of monoclonal anti-idiotypic antibodies as surrogate immunogens to induce immune responses to two carcinoma-associated antigens, TAG-72 and CEA. In this report, two anti-idiotypic antibodies to mAb CC49 and CC83 recognizing TAG-72 and CEA respectively, were generated and characterized. These anti-idiotypic antibodies were demonstrated by immunoassays (a) to react specifically with the idiotype of the monoclonal antibody (Ab1) used as immunogen and (b) to inhibit the binding interaction between the Ab1 and antigen. Anti-idiotypic antibodies AI49-3 and CAI-1 could

each induce cell-mediated immune responses specific for tumor cells that express TAG-72 and CEA, respectively.

Anti-idiotypic antibodies offer a unique opportunity to induce specific immune responses to monoclonal-antibody-defined epitopes known to be tumor-associated, thus eliminating possible cross-reactive responses to other epitopes on an antigen that may be found in normal tissues. The CEA-related antigen, non-specific cross-reacting antigen, found on the surface of human granulocytes is one such example. In addition, the use of anti-idiotypic antibodies could alleviate the problems of purifying large quantities of antigen; for example, the currently available source of CEA is liver metastases of human colon carcinoma tumors, or xenografts grown in athymic nude mice. Moreover, we have found that CEA isolated in this manner varies extensively from lot to lot resulting in heterogeneity in content (data not shown). Recombinant expression systems that produce large quantities of full-length, completely glycosylated CEA have not been developed. At this time, only very small amounts of TAG-72 have been purified from tumor xenografts in a multistep mAb column affinity purification procedure [46]. The gene coding for the protein backbone of the TAG-72 mucin has not been cloned, so no recombinant expression system is available.

"Mapping" of epitopes on an antigen by competitive binding analysis with monoclonal antibodies sometimes cannot distinguish between antibodies that bind to identical epitopes, overlapping sites or that bind separate sites of the antigen but cross-compete because of spatial hindrance. Reciprocal antibody competition binding analysis of a panel of anti-TAG-72 mAb suggested that mAb CC49, CC112, CC40, and CC50 recognize similar or adjacent epitopes on the TAG-72 molecule as determined by competition assays [27]. However, all seven radiolabeled anti-idiotypic antibodies, A149-1–7, reacted uniquely with mAb CC49 (Ab1) and not with the other CC mAb. These data defined the paratope of CC49 as being distinctive from that of those mAb that recognize similar epitopes as well as those that bind to divergent epitopes of the TAG-72 molecule. Likewise, although the COL antibodies (COL 1, 4, 6, 7, and 11) were shown to recognize identical or very similar epitopes of CEA by competition RIA [26], the anti-idiotypic antibodies demonstrated that the paratope of COL-1 is distinct from the rest. These Ab2 reagents thus recognize private epitopes found only on mAb CC49 and COL-1, respectively, and can be utilized as unique identifiers of only those idiotypes.

Attempts to generate TAG-72 or CEA-specific humoral responses by immunization of rodents with the Ab2 were unsuccessful; no antigen-specific antibody reactivity was observed in either system. One explanation may be that pre-existing Ag+ and Id+ B Cell clones are either absent or present only in very small clones [24]. Evidence now exists that the concept of internal image conformation for anti-idiotypic antibodies may only apply in special circumstances [24]. Most of the induced antigen-specific responses can be explained by the anti-clonotypic stimulation of Id+ B cell clones that are either primed by disease or those that already existed and were committed to producing the antigen-specific immune response. A second possible explanation for this observation may be that there is a modulation of Ab3 humoral immune responses against antigen that may have been below the level of detection at the time of serum testing. Finally, these Ab2 may not be entirely paratopic in nature: they may not contain enough contact residues to the paratope of the idiotype to induce an antigen-specific Ab3 antibody response [24]. An explanation for the TAG-72 system might be that it is difficult to generate humoral immunity to a carbohydrate epitope employing the protein of the Ab2 immunoglobulin. However, humoral Ab3 responses have been demonstrated that recognize polysaccharide epitopes of bacterial antigens [51].

At this time, the mechanism for cellular immune recognition of TAG-72 remains unknown. CC49 has been shown to recognize a carbohydrate epitope [27]. Antigen-specific T cell immunity against a carbohydrate moiety induced by an Ab2 is an unexpected observation since it is believed that carbohydrate itself can not induce T cell immunity. On the other hand, full characterization of the molecule may reveal that mAb CC49 (Ab1) recognizes a partially glycosylated epitope. This would allow the anti-idiotype to contain a similar sequence that could be processed and presented by antigen-presenting cells to induce T cell immune responses to TAG-72 on the surface of tumor cells. Cytolytic T cells have been described that recognize a known peptide on a mucin molecule, MUC-2 [1].

Utilizing a DTH assay, several laboratories have reported that anti-idiotypic antibodies could induce cellular immunity in both murine and human tumor systems [29, 42]. In the studies reported here, AI49-3 could induce a DTH response in mice to TAG-72 on the surface of the human ovarian carcinoma ascites cell line, OVCAR-3. In this assay we utilized xenogeneic tumor cells because no syngeneic tumor model exists at this time. Mice do not naturally express TAG-72 on their cells. However, repetition of experiments and the use of many controls as well as statistical analysis confirmed that the DTH responses we observed were not likely to be due to xenogeneic responses.

The beneficial responses induced by anti-idiotypic antibodies have been frequently reported in both experimental systems and in some human clinical investigation. Many anti-idiotypic antibodies have been described that elicit humoral Ab3 responses to tumor-associated antigen in rodent and human antigen systems. This has classically been used as a criterion for proceeding with antitumor effects in model systems or for going on to clinical trials. However, one anti-idiotypic antibody that could not induce Ab3 antibodies to a syngeneic antigen associated with a rat sarcoma could induce an antitumor effect [7]. However, the same anti-idiotypic antibody emulsified in adjuvant could induce Ab3 but could not inhibit tumor growth. Other anti-idiotypic antibodies have previously been demonstrated to elicit DTH responses to tumor antigens [29, 42]. One of these reported Ab2 could inhibit tumor growth in syngeneic rodent model systems [42]. In humans, the development of an antiidiotypic antibody response in patients administered an antitumor-associated antigen mAb to colon carcinoma has been reported to correlate with clinical improvement and long remission from disease [25, 54]. Furthermore, patients administered the same mAb demonstrated specific

DTH responses to the Ab2 that were reported to correlate with complete remission from disease (albeit in a few cases) [35]. Clinical trials with Ab2 are currently ongoing for melanoma [4, 36] and colorectal carcinoma patients [18]. To date, few clinical trials have drawn these types of correlations.

Many anti-idiotypic antibodies have been described to monoclonal antibodies that recognize CEA [3, 8, 13, 30, 37, 51]. The majority of these studies describe Ab2 species that induce Ab3 humoral immune responses specific for CEA [3, 8, 13, 30, 51] characterized by Western blot [13], immunoprecipitation [3] and immunoassays [3, 8, 13, 30, 51], as well as by immunohistochemical staining of colon carcinoma tissue sections [3]. One study has reported an Ab2 to an anti-CEA mAb that can induce a cell-mediated immune response [8]. In this study, tumor-infiltrating lymphocytes (TIL) obtained from colon carcinoma patients and stimulated in vitro with anti-idiotypic mAb were shown to proliferate in response to purified CEA. No proliferation was observed to CEA in those TIL stimulated with a control immunoglobulin. The studies reported here are the first to describe an Ab2 initiating a delayed-type hypersensitivity cellular immune response to CEA-expressing cells. This is also the first report describing anti-idiotypic antibodies to an anti-TAG-72 mAb (CC49); one of these mAb was also shown to mediate a cellular immune response. Cell-mediated immune responses such as DTH have been implicated in playing a role in tumor rejection. Therefore, these anti-idiotypic antibodies may be useful as potential immunogens for active specific immunotherapy protocols of a range of human carcinomas.

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Appendix 3 (handwritten)

CGM1a antigen of neutrophils, a receptor of gonococcal opacity proteins

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ABSTRACT *Neisseria gonorrhoeae* (GC) or *Escherichia coli* expressing phase-variable opacity (Opa) protein (Opa$^+$ GC or Opa$^+$ *E. coli*) adhere to human neutrophils and stimulate phagocytosis, whereas their counterparts not expressing Opa protein (Opa$^-$ GC or Opa$^-$ *E. coli*) do not. Opa$^+$ GC or *E. coli* do not adhere to human lymphocytes and promyelocytic cell lines such as HL-60 cells. The adherence of Opa$^+$ GC to the neutrophils can be enhanced dramatically if the neutrophils are preactivated. These data suggest that the components binding the Opa$^+$ bacteria might exist in the granules. CGM1a antigen, a transmembrane protein of the carcinoembryonic antigen family, is exclusively expressed in the granulocytic lineage. The predicted molecular weight of CGM1a is ~30 kDa. We observed specific binding of OpaI$^+$ *E. coli* to a 30-kDa band of polymorphonuclear leukocytes lysates. To prove the hypothesis that the 30-kDa CGM1a antigen from neutrophils was the receptor of Opa$^+$ bacteria, we showed that a HeLa cell line expressing human CGM1a antigen (HeLa-CGM1a) bound Opa$^+$ *E. coli* and subsequently engulfed the bacteria. Monoclonal antibodies (COL-1) against CGM1 blocked the interaction between Opa$^+$ *E. coli* and HeLa-CGM1a. These results demonstrate that HeLa cells when expressing the CGM1a antigens bind and internalize OpaI$^+$ bacteria.

Neisseria gonorrhoeae (GC), the etiologic agent of gonorrhea, can adhere to and penetrate mucosal epithelial cells (1, 2) and attain access to submucosal sites. Much of the knowledge of these interactions are based the *in vitro* infection model employing human fallopian tubes (3, 4). In this system GC pili are required for the initial adherence (5). Subsequent human volunteer studies have confirmed that pili are essential for virulence (6). In the fallopian epithelial model the distant pili-mediated attachment converts over several hours to a close attachment that is believed to be dependent on a family of outer membrane proteins, the phase-variable opacity (Opa) proteins (4). Inoculation of volunteers has indicated that Opa$^+$ gonococci are strongly positively selected in the experimental infection (7, 8). In gonococcal strain MS11, this family consists of 11 unlinked *opa* genes whose sequences are known (9). To study the role of the different Opa proteins seven Opa proteins genes have been cloned and expressed in *Escherichia coli*. In terms of cellular location, protein conformation and their interaction with human cells Opa$^+$ *E. coli* closely mimic the Opa$^+$ GC (10). Additionally, this also overcame the problem of antigenic variation and unstable expression of Opa proteins in GC. One distinct Opa protein, OpaA is correlated with adherence and subsequent internalization of GC by cultured epithelial cell lines notably Chang conjunctival cells (11–13). Moreover, it has been shown that the interaction of OpaA GC with epithelial cells involves binding to heparan sulfate on the cell surface, and that the interaction is heparin-inhibitable (14, 15). In the case of Chang cells GC-bearing Opa proteins other than OpaA do not invade the cells. However, this does not apply to all epithelial cell lines. The OpaG1 protein from strain F62 GC expressed in *E. coli* DH5α promotes attachment and invasion of ME180 cervical carcinoma cells (16). By dendrogram analysis of Opa proteins the OpaG1 protein does not belong to the same branch as MS11 OpaA (13). We have shown that OpaI expressed by *E. coli* HB101 (pEXI) also is able to adhere to ME180 cells, and that this interaction is not inhibitable with heparin (data not shown).

A major property of Opa proteins is the ability to stimulate adherence and nonopsonic phagocytosis of the Opa$^+$ bacteria by polymorphonuclear leukocytes (PMN). This increased association with human neutrophils by Opa$^+$ GC was first observed by Swanson *et al.* in 1975 (17, 18). Subsequently, several other groups demonstrated that the Opa protein mediated interaction to PMN in an opsonin-independent manner (19–21). Characteristically, some Opa proteins promote strong PMN phagocytosis such as OpaI in MS11, and other Opa proteins elicit intermediate interaction. However, OpaA GC do not stimulate PMN phagocytosis and behave like Opa$^-$ organisms (10, 13). Although Opa$^+$ GC adhere to and stimulate phagocytosis by PMN, they do not adhere to human lymphocytes and HL-60 cells (22). Farrell *et al.* (22) also noted that the interaction of Opa$^+$ GC with neutrophils could be enhanced dramatically if the PMN were preactivated with PMA and suggested that the receptors for Opa proteins were stored in secondary granules.

There has been little investigation of the biochemistry of the PMN–Opa protein interaction. It has been reported that Opa$^+$ GC bind to a 19-kDa protein of unknown identity when PMN membrane or secondary granules are separated by SDS/PAGE and transferred to nitrocellulose (23). We have found that OpaI-expressing *E. coli* are able to bind to a 30-kDa surface protein of PMN. Acting on the possibility that this protein might be CGM1a, a protein of the carcinoembryonic antigen (CEA) family, which is in that molecular weight range and is expressed only by mature PMN, we tested transfected HeLa cells expressing this antigen and found that they permitted adherence of pEXI and that the bacteria were internalized.

MATERIALS AND METHODS

Bacterial Strains, mAbs, and Cell Lines. Recombinant *opa* genes from *N. gonorrhoeae* MS11 were expressed in *E. coli* HB101 as described (10). The designations of Opa proteins of both GC and *E. coli* are based on papers of Swanson *et al.* (7) and Belland *et al.* (10). *E. coli* HB101 containing the vector pGEM-3Z is designated as pGEM. *E. coli* HB101 expressing OpaA, OpaB, OpaC, and OpaI proteins are designated as pEXA, pEXB, pEXC, and pEXI, respectively. Suspensions were prepared from bacteria grown for 16–20 h at 37°C on Luria–Bertani plates containing 50 µg/ml carbenicillin. *E. coli* strain HB101 does not express type I fimbriae. For the

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Abbreviations: GC, *Neisseria gonorrhoeae*; PMN, polymorphonuclear leukocytes; Opa protein, phase-variable opacity protein; CEA, carcinoembryonic antigen; NCA, nonspecific crossreacting antigen.

coinfection experiment a nalidixic acid resistant mutant f HB101 expressing the OpaA protein was employed.

COL-1 mAb, specific f r CGM1 and CEA, was kindly donated by Zuorong Shi (Zymed). IB4 mAb, specific for CD18, was generously provided by Sam Wright (The Rockefeller University).

Chang conjunctival and HL-60 cell lines were purchased fr m the American Type Culture Collection. HeLa-CGM1a cells were constructed by transfecting HeLa cells with CGM1a cDNA, and selected for CGM1a antigen expression on the cell surface (24). HeLa-Neo cells are HeLa cells that were transfected with the neomycin-resistance gene only. These HeLa cell lines were kindly provided by Fritz Grunert (Institute for Immunobiology, Albert–Ludwigs University, Freiburg, Germany).

Adherence and Internalization Assays. All cell lines were cultured in RPMI 1640 medium (GIBCO/BRL) with 10% fetal calf serum (HyClone). The HL-60 cell were maintained in RPMI 1640 medium containing 10% fetal calf serum, 16 μg/ml of serine, 8.4 μg/ml of asparagine, and 16.8 μg/ml of glutamine. For adherence assays, cells were grown to confluence ($\approx 2 \times 10^5$ cells per well) in 24-well culture plates (Falcon), and washed twice with serum-free RPMI 1640 medium. *E. coli* were suspended in RPMI 1640 medium to the desired OD_{540}. Bacterial suspension (0.5 ml) was added to each well. The plates were incubated at 37°C with 5% CO_2 for 3–6 h. Experiments were terminated by washing with 1 ml of serum-free RPMI 1640 medium for 2 min on an orbital shaker at 110 rpm. The wash procedure was repeated three times. Adherent bacteria were counted by suspending the cells in PBS containing 0.5% saponin (Calbiochem) and plating dilutions on Luria–Bertani-agar medium containing 50 μg/ml of carbenicillin. The level of adherence of *E. coli* to cells was calculated by determining the colony-forming units associated with the host cell monolayers. Internalization assays were done in a similar fashion, but following the period of bacterial interaction with the cells, the monolayer was washed twice and incubated for 90 min with 1.5 ml of RPMI 1640 medium/fetal calf serum (5%) supplemented with gentamicin (GIBCO/BRL) at a final concentration of 100 μg/ml. This concentration was capable of killing >99.99% of either *E. coli* or GC in the absence of epithelial cells. For adherence inhibition assays, the bacteria were added as a suspension in RPMI 1640 medium containing desired concentrations of heparin (30 μg/ml) or antibodies (25 μg/ml). The experiments were performed in duplicate or triplicate.

Interaction with PMN. PMN from 14 ml of whole human blood were purified by centrifugation through Polymorphprep (GIBCO/BRL). The purified PMN were suspended in dPBS (PBS containing 5 mM $MgCl_2$ and 1 mM $CaCl_2$) at concentration of 1×10^6 per ml. PMN suspensions (0.5 ml) were added to glass coverslips (10 mm diameter) in 24-well plates and preincubated at 37°C, with 5% CO_2 for 45 min to allow the neutrophils to adhere to the glass surface (13). After washing once with dPBS to remove the nonadherent PMN, 500 μl of Opa$^+$ *E. coli* suspensions (OD_{540} = 0.04) were added and allowed to incubate for 90 min at 37°C with CO_2. Nonadherent neutrophils and *E. coli* were removed by washing three times with dPBS, and then the PMN monolayers were fixed with 1% glutaraldehyde in dPBS containing Giemsa stain. The number of bacteria (adherent and internalized) per PMN was determined by microscopy by counting the *E. coli* associated with 100 neutrophils.

Preparation of Biotinylated PMN and HL-60 Cell Lysate. Purified PMN were suspended in 10 ml of dPBS at a concentration of 1×10^6 per ml, transferred to a 10-cm glass Petri dish, and incubated at 37°C with 5% CO_2 for 15 min to allow attachment to the Petri dish. Thereafter, 1 mg of *N*-hydroxysuccinimide-LC-biotin (Pierce) and phorbol 12-myristate 13-acetate (100 ng/ml) (Calbiochem) were added to

the Petri dish, and incubated at 37°C with CO_2 for 45 min. The reaction was st pped by washing the PMN m n layers three times with buffer containing 50 mM Tris and 150 mM NaCl at pH 7.4. The attached PMN were extracted with 3.5 ml of 50 mM Tris (pH 7.4), 150 mM NaCl, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 50 units/ml aprotinin, and 2% Triton X-100 for 30 min. The extracts were centrifuged for 5 min and the supernatants were stored at -20°C for future use. Since HL-60 cells do not bind to glass Petri dishes, the preparations of biotinylated HL-60 cells were performed in a 15-ml centrifuge tube in place of a glass Petri dish.

Binding of Specific Components from PMN By Opa$^+$ *E. coli*. Opa$^-$ or Opa$^+$ *E. coli* (pGEM or pEXI) were suspended at OD_{540} = 0.8 in 1 ml of 0.05 M Tris/150 mM NaCl buffer containing 2% BSA, 1 mM phenylmethylsulfonyl fluoride, and 10 μg/ml leupeptin and 50 units/ml aprotinin. Fifty microliters of biotinylated supernatant of PMN or HL-60 was added the bacterial suspension, and incubated at 37°C for 120 min with gentle shaking. Bacterial suspensions were pelleted. The bacterial pellets were used for Western blot analysis.

SDS/PAGE and Western Blot Analysis. Gel electrophoresis and Western blot analysis were as described (25). The Immobilon-P membrane (Millipore) with transferred biotinylated bacterial–PMN lysates was incubated in 0.05 M Tris at pH 7.4, 150 mM NaCl buffer containing 5% vitamin free-casein (Sigma) for 4 h at room temperature or overnight at 4°C. The blots were washed once with PBS-Tween (PBS containing 0.05% of Tween-20), and reacted with extravidin-conjugated peroxidase (Sigma) diluted 1:20,000 in PBS-Tween with 0.5% casein. After incubation at room temperature for 1 h, the blots were washed three times with PBS-Tween with shaking. Each wash was for 40 min. The biotinylated proteins were detected by enhanced chemiluminescence (Amersham).

RESULTS

Two Distinct Interactions of Opa$^+$ *E. coli* with Epithelial Cells and PMN. The OpaA protein expressed by *N. gonorrhoeae* MS11 promotes not only adherence but also internalization by epithelial cells (11, 12) and this interaction is inhibitable with heparin (14, 15). pEXA also adhere to Chang cells and the interaction is inhibited by heparin (Fig. 1A). pEXI adhere less well to Chang cells and this interaction is unaffected by heparin. In contrast, with PMN pEXA adhere poorly while pEXI adhere avidly and the interaction with pEXI is not inhibited by heparin (Fig. 1B). These data confirm that there are two distinct interaction mechanisms promoted by Opa proteins with epithelial cells and PMN.

Opa$^+$ *E. coli* Binds a 30-kDa Band from PMN Lysate. Among the Opa proteins, OpaI, whether expressed by GC or by *E. coli* (pEXI), shows the strongest association with PMN (10). HL-60 cells are unable to support adherence by Opa$^+$ GC (22) and were used as a negative control. We examined whether OpaI$^+$ bacteria bind to a specific protein from PMN lysed with Triton X-100. Activated and surface-biotinylated PMN or HL-60 cells were lysed in 2% Triton X-100, and then Opa$^-$ *E. coli* (pGEM) and pEXI were used to extract components from the lysates. Fig. 2 shows that pEXI specifically bound a 30-kDa band that was not seen with the control strain pGEM. Neither pEXI or pGEM bound proteins in the HL-60 lysates.

OpaI$^+$ *E. coli* Adheres to HeLa Cells Expressing CGM1a Antigens, But Not the HeLa Control Cells. Based on the molecular weight of about 30 kDa and the restricted expression in mature granulocytes we speculated that the component responsible for PMN and pEXI interaction might be CGM1a. This protein is a member of the CEA family, is restricted to the granulocytic line and is about 30 kDa in size. We used a stably transfected CGM1a HeLa cell line (HeLa-CGM1a) to test this hypothesis, and the HeLa cell line transfected only with the
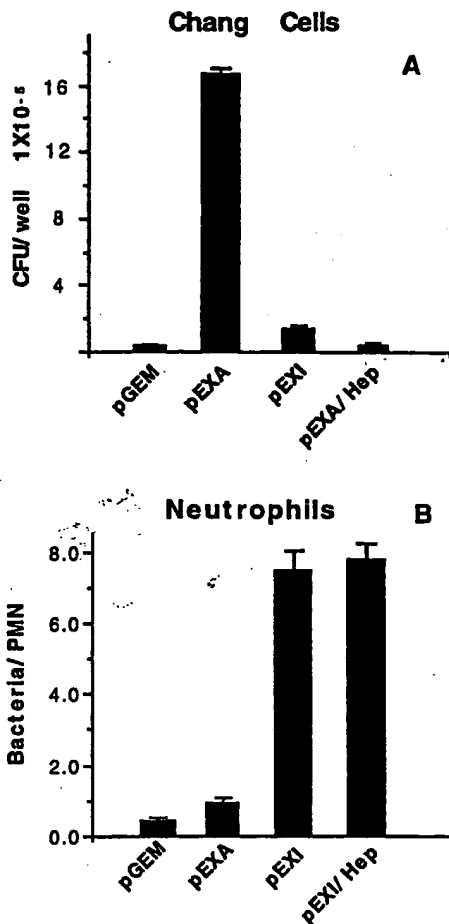
FIG. 1. Interaction of Opa⁺ E. coli with epithelial cells and neutrophils. (A) Opa⁻ or Opa⁺ E. coli were incubated with Chang conjunctival monolayers. pEXA adhere to the epithelial cells best. This interaction is inhibited by addition of heparin. pGEM and pEXI adhere at a negligible level. (B) Bacteria were incubated with PMN for 90 min. pEXI showed the highest level of adherence to PMN and this interaction was not inhibited by addition of heparin. Bars = SEM.

vector (HeLa-Neo) served as a control. As shown in Fig. 3A, there was no adherence of pGEM to the cell lines, but pEXA adhered to both cell lines. pEXI attached to HeLa-CGM1a cells only. Furthermore, the OpaA protein-mediated adherence could be blocked by soluble heparin in both cell lines, but the adherence of pEXI to HeLa-CGM1 was not influenced by heparin. Qualitatively similar results were obtained when the adherence of Opa⁻, OpaA⁺ and OpaI⁺ GC to the transfected HeLa cells was determined (Fig. 3B) CGM1a-HeLa also bound OpaB⁺ and OpaC⁺ E. coli (pEXB and pEXC) and their corresponding Opa⁺ GC to a lesser extent (data not shown).

The Interaction of OpaI⁺ E. coli to HeLa-CGM1a Cells Was Blocked by Anti-CGM1 mAb. We investigated whether a specific mAb to CGM1 could inhibit this interaction. COL-1 mouse mAb (IgG2a) is specific for CGM1, and does not react with nonspecific crossreacting antigen (NCA), BGP, and CGM6 antigens which are expressed by PMN (27) (see Discussion for description of these antigens). Anti-CD18-specific mAb, IB4 (IgG2a), was employed as a c ntrol antibody. Only COL-1 antibody inhibited the interaction f OpaI⁺ E. coli (pEXI) to HeLa-CGM1a (Fig. 4). COL-1 mAb did not inhibit the OpaA mediated adherence to HeLa-CGM1a cells (data not shown). This antibody could not be examined f r effects on the adherence of pEXI to PMN since it rapidly caused significant changes of morphology of PMN.
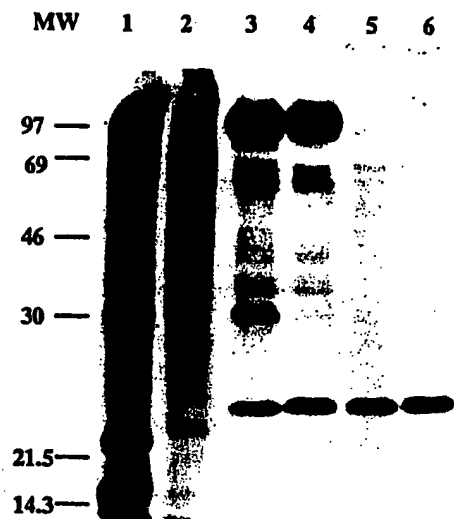

FIG. 2. pGEM and pEXI reacted with surface biotinylated PMN and HL-60 cells. PMN or HL-60 cells were lysed with Triton X-100 and mixed with bacteria. After incubation the bacteria were recovered by centrifugation, lysed, the lysates subjected to SDS/PAGE and transferred to Immobilon-P membrane. Biotinylated proteins were detected by staining with extravidin-conjugated peroxidase and chemiluminescence. PMN total lysate (lane 1) and HL-60 cell total lysate (lane 2). Proteins recovered from PMN lysate with pEXI (lane 3) and pGEM (lane 4). Proteins recovered from HL-60 cells lysate with pEXI (lane 5) and pGEM (lane 6). pEXI bind a 30-kDa band specifically from PMN lysates (lane 3). The 23-kDa protein recognized by extravidin is probably the biotin carboxy carrier protein found in E. coli (26).

The Internalization of OpaI⁺ E. coli by HeLa-CGM1a. Once CGM1a was shown to be the receptor of adherence for OpaI protein, we determined whether this interaction would promote invasion of the HeLa-CGM1a cells. As shown in Fig. 5, pGEM (Opa⁻) could not adhere to or enter the HeLa-CGM1a. In contrast, HeLa-CGM1a bound and strongly engulfed pEXI (OpaI⁺). Almost 30% of HeLa cell-associated bacteria were gentamicin resistant. pEXA (OpaA⁺) adhered to HeLa-CGM1a (Fig. 5A), but were not internalized (Fig. 5B). When coinfected with pEXI, pEXA still was unable to invade the HeLa-CGM1a cells (data not shown). This indicates that the bacteria need to bind to a specific receptor to activate the internalization system and this is distinctly different from the macropinocytosis reported for Salmonella subspecies (28). The invasion by pEXI was confirmed by electron microscopy. Both surface adherent bacteria (Fig. 6A) as well as bacteria deep in the cells in a membrane bounded compartment were seen (Fig. 6B).

DISCUSSION

Clinical GC isolated either from the male urethra or from the cervix of an infected female (except at the time of menses), are most often Opa⁺ (29–31). Male volunteers inoculated intraurethrally with Opa⁻ strains of GC, when they became infected, shed primarily Opa⁺ variants of most Opa protein types (7, 8). Furthermore, a recent study showed high level of Opa-specific antibody and protection against gonococcal salpingitis (32). Taken together, these data suggest strongly that in vivo expression of Opa proteins plays an important role in gonococcal pathogenesis. Recent work from two independent laboratories demonstrated that the OpaA protein of MS11 utilized heparan sulfate on syndecan glycoproteins as receptor molecules to interact with epithelial cells (14, 15). This interaction results in the internalization of OpaA GC into epithelial cells (15). In contrast, PMN lack receptors for OpaA, but have receptors for other Opa proteins (10, 13) and these seem to be stored within secondary granules (22).
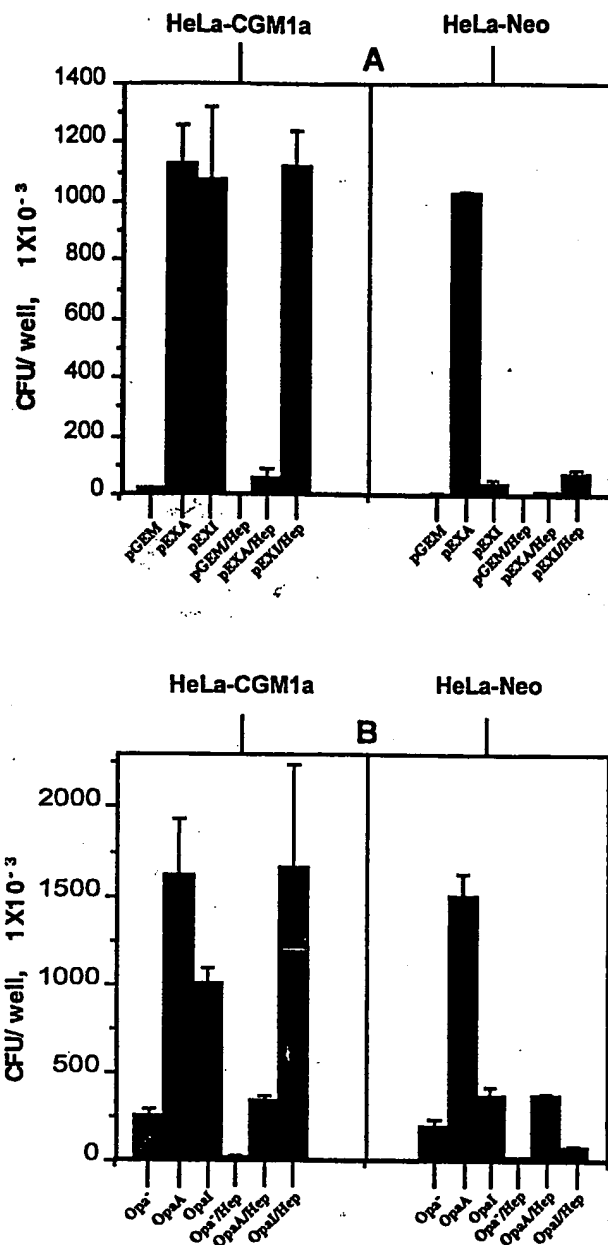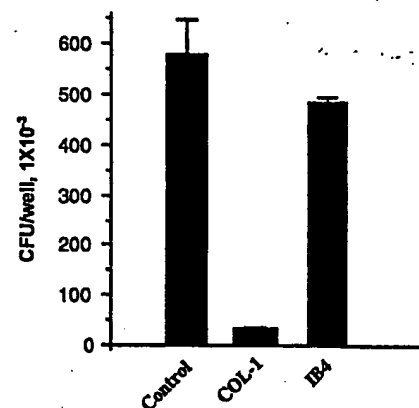
FIG. 4. Inhibition of CGM1a mediated adherence by antibody. Addition of COL-1 antibody inhibited adherence of pEXI to HeLa-CGM1a. IB4 antibody had no effect when added to the same final concentrations (25 μg/ml). The interaction of pEXI with HeLa-CGM1a was for 2.5 h.

CGM1 antigens of neutrophils belongs to the family of CEA. Studies trying to identify tumor-specific antigens led t the discovery of CEA, a 180-kDa tumor-associated cell-surface glycoprotein on colon cancer cells (33, 34). Subsequently, it
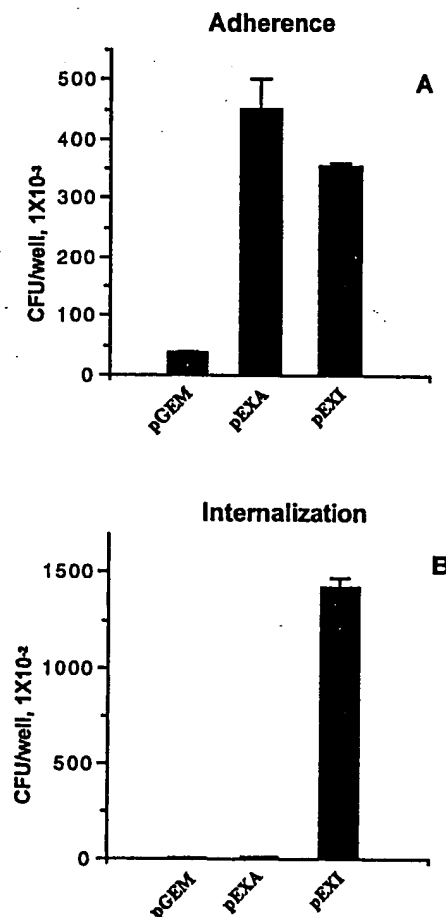

FIG. 3. Interactions of Opa⁺ *E. coli* and GC with HeLa-CGM1a cells. Opa⁺ *E. coli* (A) and GC (B) were incubated with HeLa-CGM1a and HeLa-Neo cells in RPMI medium 1640 buffer with or without soluble heparin (Hep. 30 μg/ml). pEXI adhere to the CGM1a cells but not the control cells. OpaA mediated adherence is inhibited by heparin, but the adherence of pEXI to CGM1a is not influenced by soluble heparin. Similar results were observed with OpaA and OpaI GC.

The current study demonstrates that human CGM1a appears to be a receptor for the interaction of Opa⁺ GC with PMN. Attention was directed to the role of the CGM1a antigen by the observation that OpaI⁺ *E. coli* bound specifically a ~30-kDa protein band, which correlated well with the predicted molecular weight of CGM1a. The CGM1 antigens are only expressed in neutrophils, not in human monocytes, lymphocytes and HL-60 cells (24). Transfected HeLa cells expressing CGM1a were able to avidly bind and internalize OpaI⁺ *E. coli*, but not *E. coli* containing only the pGEM plasmid vector. This interaction did not occur with HeLa cells transfected with the neomycin-resistance gene only. The interaction was not inhibited by addition of heparin, but was strongly inhibited by a mAb specific for CGM1.

FIG. 5. The internalization of pEXI by HeLa-CGM1a cells. pGEM, pEXA, and pEXI at OD₅₄₀ = 0.04 were incubated with HeLa-CGM1a cells in RPMI 1640 medium for 6 h. Unbound cells were removed by replacing the supernatants with fresh RPMI medium 1640 (1 ml per well per wash) at 3-h intervals. The adherent and intracellular *E. coli* were distinguished by incubation with gentamicin. Only pEXI were recovered in large numbers following gentamicin treatment, although both pEXA and pEXI adhered to the HeLa-CGM1a.

FIG. 6. Transmission electron micrograph of internalization of pEXI by HeLa-CGM1a cells. (A) OpaI⁺ E. coli are shown in the early stage of interaction with HeLa-CGM1a cells. (B) The internalized E. coli are clearly enclosed within vesicles as indicated with arrows. (Bar = 1 μm.)

was found that antibodies to CEA also reacted with normal cells and identified a number of closely related, crossreacting antigens, initialy termed NCAs (35–37). The CEA gene, located in human chromosome 19 (38), is a member of a family of >17 expressible closely related genes (39) that belong to the Ig gene superfamily (40). The human CEA family, recognized by CD66 and CD67 antibodies, consists of two major subfamilies, one termed the CEA subgroup, containing CEA, NCA, biliary glycoprotein (41), CGM1 (CGM1a, CGM1b, and CGM1c) (24, 42), CGM2, CGM6, and CGM7 (CGM = CEA gene family member). The second subfamily consists of the pregnancy-specific glycoproteins (43–45).

Our studies have not addressed the role of any other member of the CEA family as Opa protein receptors. Virji et al.* recently reported that COS cells transfected with CD66a antigen—i.e., binary glycoprotein—caused opacity protein specific adherence of N. meningitidis. This activity was depen-

dent on the immunoglobulin-like N-terminal domain This IgV-like portion of the molecule is strongly conserved among different members of the CEA family and CGM1 consists solely of this domain. Taken together with our results presented for CGM1a neutrophil antigen. it would appear that several CEA family members may serve as receptors. CD66a antigen is expressed in many tissues including urogenital epithelial cells (46). It remains to be determined whether the opa-mediated interaction recognizes the CEA protein structure or a glycosylation pattern shared by this family of proteins. It has been previously reported that the type I fimbriae of E. coli attach to purified CEA, binary glycoprotein, and NCA (47), and that mannose-inhibitable adherence to neutrophils was dependent on the presence of NCA-50 (48). It has been demonstrated that this reactivity is due to high mannose type oligosaccharides found on the three glycosylation sites in the N-terminal domain of this antigen (49).

Internalization of microorganisms into either professional or nonprofessional cells by phagocytosis require the reorganization of the actin-based cytoskeleton. This actin assembly is initiated by signals arising from the interaction of phagocytosis-promoting receptors on the cell surface with ligands on the surface of the microorganisms such as invasin on Yersinia pseudotuberculosis (50, 51). Several receptors have been identified to mediate binding and ingestion of phagocytic particles. The best-characterized receptors are the opsonin-recognizing receptors including the various types of receptors for the Fc portion of IgG ($Fc_\gamma Rs$) (52). After the opsonized bacteria bind the Fc_γ RIII, the tyrosine-activation motif on the cytoplasmic domain of Fc_γ RIII is activated by phosphorylation. These phosphorylated tyrosine residues within tyrosine-activation motifs, can recruit another tyrosine kinase, Syk, whereupon it becomes activated. Activation of Syk as a result of clustering of receptors by antigen, in the case of T and B cells, or by immune complexes, in the case of Fc_γ and Fc_ϵ receptors, leads to phagocytosis or actin polymerization (53). In fact, CGM1a has a cytoplasmic domain where tyrosines lie within a sequence context ($YX_2LX_7YX_2M$) (24), which is similar to the consensus sequence ("YLYL" motif: $YX_2LX_7YX_2L/I$) found in the cytoplasmic domain of molecules of multichain immune recognition receptors (54). Since the cytoplasmic domains of CGM1a contains a tyrosine-activation motif-like motif, it is possible that in the HeLa transfectants CGM1a may have acted not only as receptor for the adherence of OpaI⁺ E. coli, but also as a signal transducing molecule initiating the internalization of the bacteria. The role of CGM1a in PMN physiology remains to be explored.

The biological role of the GC Opa proteins is now becoming clearer as the eukaryotic ligands for these proteins are being elucidated. It has been established that a subset of opa proteins binds to syndecans (14, 15). Syndecans serve as receptors or coreceptors for growth factors, for cell-to-cell interactions and for cell interactions with extracellular matrix components (reviewed in ref. 55). The finding that MS11 Opal as well as OpaB and OpaC bind to neutrophil CGM1a defines a new molecular basis for the adherence of GC to host surfaces. The extent that this specificity applies to other members of the CEA family of proteins and the role that these interactions may have in signaling the internalization of GC by both neutrophils and epithelial cells will prove a fertile area for investigation.

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PROCEEDINGS

OF THE

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December 10, 1996

Volume 93 / Number 25

Appendix 5

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re patent application of

Hans J. HANSEN et al.

Serial No. 09/253,794

Filed: February 22, 1999

For: CDR GRAFTED TYPE III ANTI-CEA HUMANIZED
MOUSE MONOCLONAL ANTIBODIES

Atty. Docket No: 018733/0852

Group Art Unit: 1644

Examiner: D. Saunders

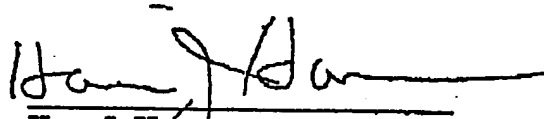### DECLARATION UNDER 37 C.F.R. §1.132

I, Hans J. Hansen, declare that:

1.   I am a co-inventor of the above-captioned application. I have held the position of Vice President, Research & Development at Immunomedics, Inc., Morris Plains, New Jersey, from 1992 to the present.

2.   On and prior to October 5, 1994, the filing date of the parent application, U.S. Serial No. 08/318,157, now U.S. Patent No. 5,874,540, of the above-captioned application, the hybridoma that produces the Class III, anti-CEA murine monoclonal antibody, MN-14, was a proprietary cell line owned and possessed by Immunomedics, Inc. Thus, the DNA encoding MN-14 was not available to the public prior to the priority date of the above-captioned application.

3.   I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further, that these statements are made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

19 May 2001
Date

Hans J. Hansen

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Atty. Docket No: 018733/0874

In re patent application of

Shiu-on LEUNG *et al.*

Group Art Unit: 1642

Serial No. 09/155,106

Examiner: C. Yaen

Filed: November 17, 1998

For: HUMANIZATION OF AN ANTI-CARCINOEMBRYONIC ANTIGEN ANTI-IDIOTYPE
ANTIBODY AND USE AS A TUMOR VACCCINE AND FOR TARGETING
APPLICATIONS

DECLARATION UNDER 37 C.F.R. §1.132

I, David Milton Goldenberg, declare that:

1.   I am the Chairman of the Board and founder of Immunomedics, Inc., where I have
been employed since 1983. I have remained Chairman since the inception of the Company, and have
at various times also been Chief Executive Officer and President. I also am the President and founder
of the Center for Molecular Medicine and Immunology (CMMI), as well as its clinical unit, the
Garden State Cancer Center, both constituting non-profit research centers located in Belleville, New
Jersey. I have been President of CMMI since 1983, and incorporated this institution in 1982.

2.   On and prior to March 20, 1996, the filing date of the priority document, U.S. Serial
No. 60/013,708, of PCT/US97/04696, filed on March 19, 1997, to which the above-captioned
application claims priority, the hybridoma that produces the rat anti-idiotype antibody (rWI2) against
a Class III, anti-CEA murine monoclonal antibody, MN-14, was a proprietary cell line owned and
possessed by Immunomedics, Inc., and researched exclusively by Immunomedics and CMMI
investigators in collaborative projects. Thus, the DNA encoding rWI2 was not available to the public
prior to the priority date of the above-captioned application.

3.   I hereby declare that all statements made herein of my own knowledge are true and
that all statements made on information and belief are believed to be true; and further, that these
statements are made with the knowledge that willful false statements and the like so made are
punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States
Code and that such willful false statements may jeopardize the validity of the application or any
patent issuing thereon.

Dec. 2, 2002
Date

David Milton Goldenberg (signature)

David Milton Goldenberg